UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

006707

MAY 11 1988

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM:

TO: Lois Rossi, PM # 21
Fungicides/Herbicides Branch
Registration Division TS-767C

THRU: R. Bruce Jaeger, Section Head
Rev. Sec. 1/Toxicology Branch
Hazard Evaluation Division (TS-769C)

THRU: Dr. T. M. Farber, Chief
Toxicology Branch
Hazard Evaluation Division (TS-769C)

FROM: D. Ritter, Toxicologist
Rev. Sec. 1/Toxicology Branch
Hazard Evaluation Division (TS-769C)

Subject: EPA # 50534-7 - CX, submission of additional toxicity data.

Sponsor: Fermenta Plant Protection Co., Painesville, OH.

Caswell: 215B.

TOX Project #: 7-0915.

The Sponsor is submitting additional toxicity data in support of CX registrations. These data are related to the Registration Standard for CX, issued in 1984. The Sponsor wants the Agency to consider these studies in our evaluation of the oncogenicity and chronic toxicity of CX. The studies have been reviewed and their DERs are attached.

1. Two Year Feeding Study in Male Mice - Final Report. # 1099-84-0077-TX-006. MRID #40243701.

   Feeding male CD-1 mice CX in diet for two years did not produce neoplasms in the stomach or kidneys at up to 750 ppm, the highest level tested. The NOEL for non-neoplastic effects was 40 ppm based on renal tubular hyperplasia. The study is rated CORE Minimum data.

2. 90 Day Rat Feeding Study in Male Rats. # 1115-85-0079-TX-006. MRID # 40243702.

   Male Fischer 344 rats were offered diets containing 0 or 175 mg/kg/day CX. Gastric and renal lesions consisting of squamous epithelial effects were noted as early as 4 days of feeding. continued feeding resulted in erosion and gastritis. At 4 days of feeding renal degenerative effects were noted, followed by renal epithelial hyperplasia, regeneration and hypertrophy. The trithiol metabolite, but not the dithiol moiety, appeared in the urine.

3. Determination of Co-valent Binding of DNA to CX in the Kidney of Male Rats. #1173-86-0096-AM-002. MRID # 40243703.

   Sprague-Dawley male rats were treated with 14-C CX or with IP 14-C-DMNA. Six hour later the renal DNA for each was determined. Only the rats receiving DMNA showed co-valent binding of renal DNA; Those receiving CX did not show co-valent binding of renal DNA.

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Reviewer: D. Ritter, Toxicologist DLR 5-6-88 Caswell #: 215B
Rev. Sec. # I/Toxicology Branch
Secondary Reviewer: R. Bruce Jaeger, Section Head
Rev. Sec. # I/Toxicology Branch

DATA EVALUATION RECORD

Study: Two Year Feeding Study in Male Mice.

MRID: 40243701

Performing Laboratory: International Research & Development Corp.
Mattawan, MI.

Author(s): N. B. Wilson and J. C. Killeen.

Study ID Number: 1099-84-007-TX-006.

Date of Study: 6/12/87

Title: A Tumorigenicity Study of Chlorothalonil in Male Mice - A Final Report.

CORE Rating: Minimum data.* Although only male mice were used, the study accomplished it original purpose of defining the effects of Chlorothalonil on kidneys and stomach at doses of 750 ppm and below. Although only the kidneys and stomachs were examined histologically, most other required tissues and organs were collected and preserved for further study if needed.

QA Statement: Statisfactory.

Conclusions: The NOEL for non-neoplastic effects is 40 ppm based on renal tubular hyperplasia. The NOEL for oncogenic effects is greater that 750 ppm in this study.

METHODS:

Purpose -
"... to determine, if possible, the no-effect level for tumorigenic effects in the kidney and forestomach in male Charles-River CD-1 mice following chronic dietary administration of technical Chlorothalonil".

* NOTE: Although rated Minimum data, this study will not support registration of products containing Chlorothalonil because it does not fulfill data requirements for such studies.

Animals -

Charles River CD-1 male mice, sixty per group were offered diets containing 10 (increased to 15 at week eighteen), 40, 175 or 750 ppm technical Chlorothalonil fortwo years. A similar group received diet only.

Husbandry - Standard GLP.

Feed and Water - Available ad libitum.

Observations -

Twice daily for morbidity and signs of toxicity. Detailed physical exams were done weekly.

Body Weights and Feed Consumption -

Initially, then weekly through week 14, then biweekly thereafter.

Diets -

Prepared at least every week and analyzed for Chlorothalonil content every week.

In-Life Measurements -

Blood was collected from ten animals per group at twelve, eighteen and twenty-four months for routine hemotological examinations. These included RBCs, HCT, WBCs and segmented neutrophils.

Post-Mortem Examinations -

All animals which were moribund or which died during the study were necropsied. The same animals (10 per dose level) used for the twelve month blood sampling were also necropsied at that time. All surviving animals were necropsied at twenty-four months. The brains and kidneys were weighed at the one year interim necropsy and at termination. The kidneys, stomachs and related lymph nodes were prepared for histopathological examination. A full complement of tissues and organs was collected and preserved in phosphate-buffered formalin solution. (See Appendix A). These were examined macroscopically. The brains and kidneys were weighed.

RESULTS:

Morbidity and Signs of Toxicity -

There was no excessive mortality that could be attributed to treatment with Chlorothalonil, and no group showed toxic signs or symptoms. All animals maintained good physical condition throughout the study.

Body Weights and Feed Consumption -

Body weights and rates of weight gain were similar among the treated and control groups. Feed consumption was not altered by exposure to Chlorothalonil.

Diets -

Analysis of samples prepared weekly or more often showed that the feed contained the intended amounts of test material.

Blood Analyses -

HCT, HGB and neutrophils were significantly decreased at 12 months in the 750 ppm group, but not at any other time or at any other dose level. This effect is not considered to be treatment-related.

Post-Mortem Examinations -

Organ Weights -

Absolute kidney weights and kidney-to-brain and kidney-to-body weight ratios were comparable to those of the control group for the 10/15, 40 and 175 ppm groups. At one year the kidney-to-body weight ratio was significantly increased for the 750 ppm group when compared to that of the control group.

The kidney-to-brain and kidney-to-body weight ratios, as well as the absolute kidney weights were significantly increased in the 750 ppm group at two years.

Gross Necropsy -

There was some enlargement of the spleens noted in animals in the control group and in the treated groups at the first year necropsy, but, since this was not seen at termination, the authors concluded that the phenomenon did not represent a compound-related effect.

Microscopic Examination -

Kidneys -

Chlorothalonil induced tubular hyperplasia and karyomegaly in the 175 and 750 ppm groups. Animals in the 750 ppm group showed an increase in the incidence of tubular hypertrophy. The NOEL for these effects is 40 ppm.

Renal cortical cysts were reported in all dose groups and the control, affecting about 50 % of all animals. This is not considered to be a treatment-related finding.

There was one tubular adenoma each in the 40 and 175 ppm groups; this was not considered to be related to dietary administration of Chlorothalonil. These lesions did not occur in mice that also showed tubular hyperplasia. The control group and the 40 ppm group each had 1/60 of malignant lymphoma; there were 2/60 of these lesions in the 175 ppm group. There were 5/60 each of malignant lymphomas in the 10/15 and 750 ppm groups. There is some question whether this represents an oncogenic effect since there is no apparent dose-relationship, and since this lesion is common in CD-1 mice.* However, the registrant should provide more recent historical control data on this lesion, particularly those derived from CD-1 mice in studies done at the performing laboratory.

Table I shows the distribution of non-neoplastic and neoplastic renal lesions in this study.

Stomach -

Histopathological examinations of the forestomachs revealed that Chlorothalonil induced hyperplasia and hyperkeratosis of the squamous epithelium. There was a clear dose-response relationship in the occurence of this lesion. Squamous papillomas were reported for the control group (2/60) and for the 175 and 750 ppm groups (1/60 and 3/60, respectively).

The authors reported the occurence of 1/60 squamous carcinomas in the 750 ppm group. See Table II.

Hyperplasia of the glandular mucosa of the stomach was reported in mice of all groups; there was a tendency for this lesion to occur more often in the treatd groups; however, there was no clear-cut dose-response seen.

1/60 instances of glandular papilloma were reported for the control group. This was the only neoplasm of the glandular stomach. See Table III for a summary of findings in the gastric mucosa.

DISCUSSION:

This study failed to show the anticipated induction of renal and gastric neoplasms associated with the dietary administration of Chlorothalonil that have been reported in previous mouse studies (MRID #00127858) and in rat studies (MRID # 00146945). Based on the findings in the chronic mouse study, and on the negative results of this study, the authors have concluded that the NOEL for renal tubular neoplasms and for tumors of the forestomach is 175 ppm. (21.3 mg/kg/day). The non-neoplastic NOEL is 40 ppm based on renal tubular hypertrophy at the high dose levels.

The study cited in support of the author's contention, that the incidence of malignant lymphoma reported in this study is common to CD-1 mice, is dated 1971. Additional historical control data (from a more recent period; e.g., 1985 to the present) on this lesion in this strain should be provided by the performing laboratory.

This study is being rated CORE Minimum data because it provides answers to questions concerning NOELs for renal and stomach lesions, and because it was a well conducted study.

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* Percy, D. H., et al. Incidence of Spontaneous Tumors in CD-1 HaM/ICR Mice. J. Nat. Canc Inst. 46:1045-65. 1971.

APPENDIX A

Representative samples of protocol designated organs and tissues were collected and placed in Carson's modified Millonig's phosphate-buffered formalin. A full complement of organs and tissues was collected from all animals.

The following organs and tissues were collected:

- Adrenal (2)
- Aorta
- Bone (femur)
- Bone marrow (sternum)
- Brain (fore, mid and hind)
- Cecum
- Colon
- Duodenum
- Epididymis (2)
- Esophagus
- Eye with Harderian gland (2)
- Gallbladder
- Heart
- Ileum
- Jejunum
- Kidney (2)
- Liver
- Lung with mainstem bronchi[a]
- Lymph nodes [mesenteric (abdominal), gastric, renal, cervical and regional[b]]
- Pancreas
- Pituitary
- Preputial gland
- Prostate
- Rectum
- Salivary gland (submaxillary)
- Sciatic nerve
- Seminal vesicle (2)
- Skeletal muscle
- Skin
- Skull
- Spinal cord (cervical, mid-thoracic and lumbar)
- Spleen
- Stomach (forestomach and fundus)[c]
- Testis (2)
- Thymus
- Thyroid/parathyroid complex
- Tongue
- Trachea
- Urinary bladder
- All tissue masses
- All gross lesions

TABLE I

INCIDENCE[a] OF SEVERAL RENAL LESIONS OBSERVED HISTOPATHOLOGICALLY IN THE TUMORIGENICITY STUDY IN MICE WITH TECHNICAL CHLOROTHALONIL

Histopathologic Finding	Study Interval, Mo.	Dietary Concentration, ppm				
		0	10/15	40	175	750
Tubular Hyperplasia	0-12	9/13	9/17	8/12	10/14	14/16
	12-24	5/47	1/43	7/48	10/46	25/44
	0-24	14/60	10/60	15/60	20/60	39/60
Tubular Hypertrophy	0-12	1/13	0/17	1/12	0/14	6/16
	12-24	1/47	1/43	1/48	1/46	4/44
	0-24	2/60	1/60	2/60	1/60	10/60
Karyomegaly	0-12	0/13	4/17	2/12	8/14	8/16
	12-24	11/47	7/43	13/48	13/46	24/44
	0-24	11/60	11/60	15/60	21/60	32/60
Cortical Cysts	0-12	2/13	0/17	3/12	2/14	2/16
	12-24	28/47	25/43	30/48	25/46	33/44
	0-24	30/60	25/60	33/60	27/60	35/60
Tubular Adenoma	0-12	0/13	0/17	0/12	0/14	0/16
	12-24	0/47	0/43	1/48	1/46	0/44
	0-24	0/60	0/60	1/60	1/60	0/60
Malignant Lymphoma	0-12	0/13	3/17	0/12	2/14	2/16
	12-24	1/47	2/43	1/48	0/46	3/44
	0-24	1/60	5/60	1/60	2/60	5/60

[a] $\text{incidence} = \frac{\text{affected animals}}{\text{number of animals examined at each study interval}}$

TABLE II

INCIDENCE[a] OF SELECTED HISTOPATHOLOGIC FINDINGS IN THE FORESTOMACH IN THE TUMORIGENICITY STUDY IN MICE WITH TECHNICAL CHLOROTHALONIL

Histopathologic Finding	Study Interval, Mo.	Dietary Concentration, ppm				
		0	10/15	40	175	750
Squamous hyperplasia/ hyperkeratosis[b]	0-12	0/13	0/17	1/12	2/14	8/16
	12-24	2/47	2/43	10/48	9/46	31/44
	0-24	2/60	2/60	11/60	11/60	39/60
Squamous papilloma	0-12	0/13	0/17	0/12	0/14	0/16
	12-24	2/47	0/43	0/48	1/46	3/44
	0-24	2/60	0/60	0/60	1/60	3/60
Squamous carcinoma	0-12	0/13	0/17	0/12	0/14	0/16
	12-24	0/47	0/43	0/48	0/46	1/44
	0-24	0/60	0/60	0/60	0/60	1/60

[a] $\text{incidence} = \frac{\text{affected animals}}{\text{number of animals examined at each study interval}}$

[b] Incidence of animals in which hyperplasia or hyperkeratosis or both findings were observed in the forestomach.

TABLE III

INCIDENCE[a] OF SELECTED HISTOPATHOLOGIC FINDINGS IN THE GLANDULAR STOMACH IN THE TUMORIGENICITY STUDY IN MICE WITH TECHNICAL CHLOROTHALONIL

Histopathologic Finding	Study Interval, Mo.	Dietary Concentration, ppm				
		0	10/15	40	175	750
Glandular hyperplasia	0-12	0/13	2/17	2/12	1/14	4/16
	12-24	8/47	13/43	21/48	5/46	16/44
	0-24	8/60	15/60	23/60	6/60	20/60
Glandular papilloma	0-12	0/13	0/17	0/12	0/14	0/16
	12-24	1/47	0/43	0/48	0/46	0/44
	0-24	1/60	0/60	0/60	0/60	0/60

[a] $\text{incidence} = \frac{\text{affected animals}}{\text{number of animals examined at each study interval}}$

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Reviewer: D. Ritter, Toxicologist DR 5-6-88 Caswell #: 215B
Rev. Sec. # I/Toxicology Branch
Secondary Reviewer: R. Bruce Jaeger, Section Head
Rev. Sec. # I/Toxicology Branch RBJ 5/6/88

DATA EVALUATION RECORD

Study: 90 Day Rat Feeding Study (males).

MRID: 40243702

Performing Laboratory: In Life phase: International Research & Development Corporation, Mattawan, NJ.

Histopathology: Experimental Pathology Laboratories, Inc., Herndon, VA.

CERTI, Versailles, France.

Author(s): W. H. Ford and J. C. Killeen, Jr.

Study ID Number: 1115-85-0079-TX-006.

Date of Study: 6/4/87.

Title: A 90-Day Feeding Study in Male Rats With Chlorothalonil.

Material Tested: Chlorothalonil;
2,4,5,6-tetrachloroisophathalonitrile.

Synonyms: Daconil; SDS-2787; DS-2787.

CORE Rating: Minimum data.* Although only males were used, the study accomplished its original purpose of detecting the earliest date at which gastric and renal lesions appear following the initiation of feeding of dietary Chlorothalonil. The study also confirms that Chlorothalonil induces lesions similar to those identified in previous feeding studies.

QA Statement: Acceptable.

Conclusions:

Chlorothalonil induces gastric and renal lesions as early as four days after commencing dietary exposure in male rats at a level of 175 mg/kg/day.

* NOTE: Although rated Minimum data, this study will not support registration of products containing Chlorothalonil because it does not fulfill data requirements for such studies.

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Conclusions:

Chlorothalonil induces gastric and renal lesions as early as four days after commencing dietary exposure in male rats at a level of 175 mg/kg/day.

* NOTE: Although rated Minimum data, this study will not support registration of products containing Chlorothalonil because it does not fulfill data requirements for such studies.

METHODS:

Animals used: Fischer 344 male rats, 90 per group.

Dosing: Chlorothalonil was administered in the diet at levels of 0 (Control) and 175 mg/kg/day (Treatment).

Husbandry: Standard GLP.

Feed and Water: Available ad libitum.

Chlorothalonil was administered in the diet of 90 Male Fischer 344 rats for up to 91 days. A similar group received untreated diet and served as controls. Observations were made daily for viability. Feed consumption and physical condition was determined weekly. Ten animals were randomly selected from each group for necropsy on study days 4 and 7, and at the end of weeks 2, 4, 6, 8, 10, 12 and after 91 days of exposure.

Urine samples were collected for 16 - 20 hours on day 4 and for the 24 hours just prior to necropsy on day 7 and at the end of weeks 2, 4, 6, 8, 10 and 12. These samples were analyzed for the di- and trithiol metabolites of Chlorothalonil.

Brain and kidney weights were recorded and any gross lesions were collected and stored in 10% neutral formalin. Gross and microscopic analyses of stomach, kidneys and gross lesions were performed.

The stomach was inflated with formalin solution and fixed. Following fixation the organ was opened along the greater curvature and the surfaces were examined. The stomach was then processed for H & E staining and examined histologically.

The right kidney was prepared for histopathological examination by H & E staining and the left kidney was prepared by Masson's Trichrome stain to demonstrate connective tissue.

Samples of the diets were analyzed weekly for Chlorothalonil content.

RESULTS:

Observations for Viability -

There was no unscheduled mortality in either group.

Body Weights -

The body weights of the Chlorothalonil-treated rats was lower by 3 - 9 % than the controls; this was a significant difference and was considered to be compound-related.

Food Consumption -

Food consumption was reduced at 4 and 7 days in the Chlorothalonil-treated rats. Later in the study the food consumption relative to body weight was similar between the groups. The early reduction was thought to be due to the irritant properties of Chlorothalonil.

Urinary Metabolites -

Only the trithiol metabolite of Chlorothalonil was found in the urine samples taken at the times of sacrifice. This appeared in a cyclic pattern, being highest (30, 36, 31 and 26 ugm/rat/day) on day 4 and at weeks 4, 8 and 12. Lesser amounts (17 and 7 ugm/rat/day) were reported for weeks 2 and 10. No trithiol metabolite was found on day 7 or on week 6. See Figure A, appended. The authors could offer no explanation for this finding.

Diet Analysis -

Diets fortified with Chlorothalonil assayed at 95.5 - 106.8 % of the intended concentration.

Necropsy -

Gross -

Treatment-related gross changes were reported for the forestomachs of Chlorothalonil-treated rats, beginning at 7 days. From day 7 to week 8 the changes were principally characterized by multifocal erosions of the non-glandular portion, with the highest incidence (9/10) being observed at day 7. This lesion was absent at 10 and 12 weeks and at termination.

Thickening of the non-glandular portion was seen at the end of week 4 and affected 7/10 rats. It was seen in virtually all the remaining Chlorothalonil-treated rats for the remainder of the study. See Table I.

The remainder of the gross post-mortem examination was essentially normal.

Organ weights -

Brain weight and brain-to-body-weight ratios were within normal limits for the control and Chlorothalonil-treated rats.

Kidney weights -

The mean absolute kidney weights, the kidney-to-body-weight ratios and the kidney-to-brain ratios were significantly increased in the Chlorothalonil-treated rats when compared to those of the controls. These increased weights were generally associated with microscopic changes in the kidneys.

Microscopic -

Kidneys (H & E stains) TABLE II.

Table II shows the occurence of major histological lesions that were reported for the kidneys which were H & E stained. Hyaline droplets were reported in both groups beginning at week 4. The authors consider this to be related to maturational events and is not treatment-related. Tubular epithelial vacuolization, nuclear pyknosis, loss of brush border, karyomegaly and tubular epithelial regeneration were reported for days 4 and 7 in the treatment group but not in the controls. Except for a low incidence of karyomegaly late in the study, these lesions did not reappear. Tubular hypertrophy appeared at week 2 in the treated animals and, except for week 8, was noted at every interval thereafter during the study. Epithelial regeneration and hyperplasia were prominent findings in the treated group beginning at day 7 and week 4, respectively.

Kidneys (Mason's Trichrome stains) TABLE III

Table III shows the tubular necrosis that was reported in both groups beginning at week 2 and that remained throughout the study. Tubular casts were reported for the treated group at 6 weeks and thereafter. Foci of basophilic cytoplasm began to appear in both groups at week 6 and thereafter. Tubular ectasis (swelling or stretching) was seen in the treated group at week 2, 8, 10 and 12, but was reduced at termination.

Stomach -

Treated animals demonstrated squamous epithelial hyperplasia and hyperkeratosis beginning at day 4 and at every period thereafter. The controls did not demonstrate these effects. Gastritis, possibly associated with erosion of the forestomach, was reported for weeks 2, 4 and 6, with fewer animals being affected thereafter.

DISCUSSION:

Feeding Chlorothalonil in the diet to male rats for ninety days resulted in the appearance of squamous epithelial effects in the forestomach as early as four days after beginning treatment. Continued feeding resulted in erosion and gastritis. Renal effects likewise appeared very early. At four days the animals demonstrated degenerative effects which were replaced by subsequent appearance of epithelial hyperplasia, regeneration and tubular hypertrophy. The trithiol metabolite of Chlorothalonil appeared in the urine of treated rats in cyclic fashion, peaking initially at four and eight weeks at urinary concentrations of about 0.1 % of the administered daily dose. This phenomenon was not explained.

Overall the study supports the author's contention that Chlorothalonil fed in the diet of male rats induces gastric and renal lesions as early as 4 days.

This study is being rated CORE Minimum data because it provides answers to questions concerning renal and stomach lesions, and because it was a well conducted study.

DISCUSSION:

Feeding Chlorothalonil in the diet to male rats for ninety days resulted in the appearance of squamous epithelial effects in the forestomach as early as four days after beginning treatment. Continued feeding resulted in erosion and gastritis. Renal effects likewise appeared very early. At four days the animals demonstrated degenerative effects which were replaced by subsequent appearance of epithelial hyperplasia, regeneration and tubular hypertrophy. The trithiol metabolite of Chlorothalonil appeared in the urine of treated rats in cyclic fashion, peaking initially at four and eight weeks at urinary concentrations of about 0.1 % of the administered daily dose. This phenomenon was not explained.

Overall the study supports the author's contention that Chlorothalonil fed in the diet of male rats induces gastric and renal lesions as early as 4 days.

FIGURE A

URINARY EXCRETION OF TRIMETHYLTHIOCHLOROISOPHTHALONITRILE
DURING THE COURSE OF A 90-DAY FEEDING STUDY
WITH CHLOROTHALONIL (175 MG/KG/DAY)

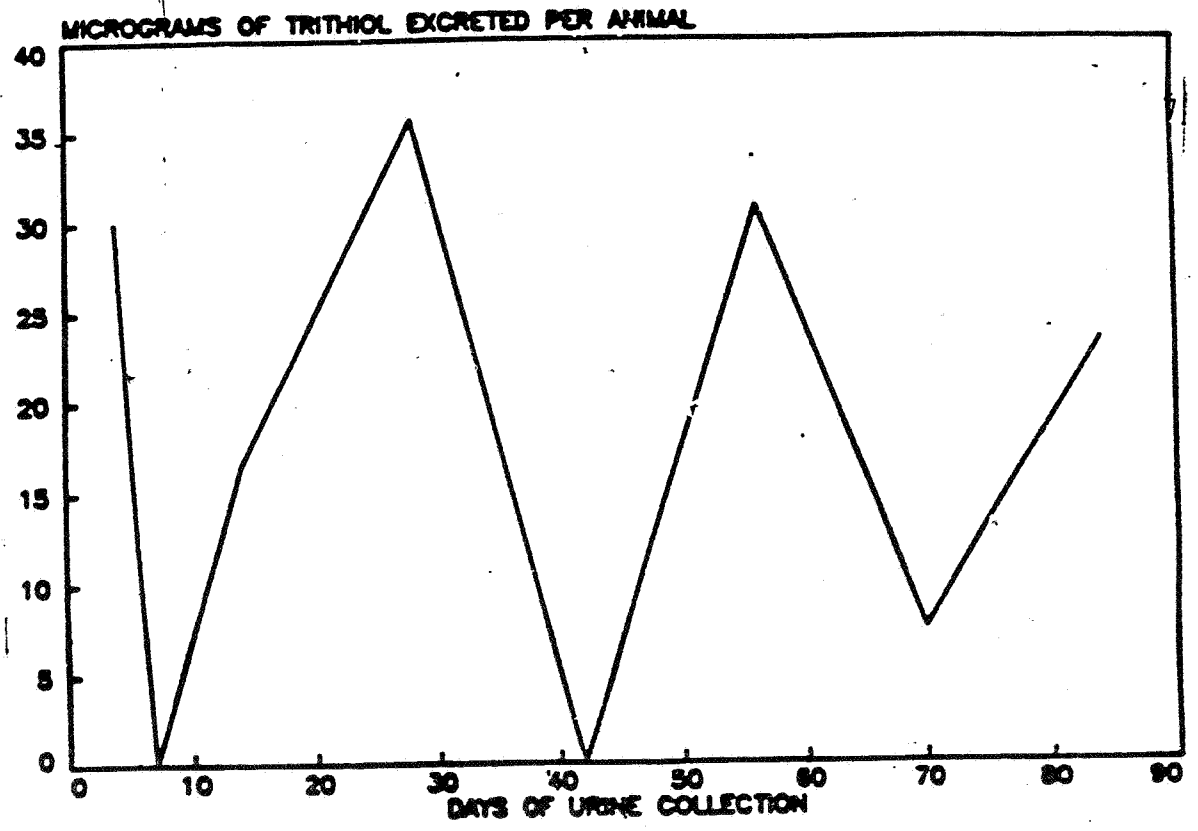

TABLE I

175 mg/kg/day
INCIDENCE OF GROSS PATHOLOGY FINDINGS
IN THE NON-GLANDULAR STOMACH

	Day		Week						
Mucosal Finding	4	7	2	4	6	8	10	12	Term.
Multifocal Erosions	0/10	9/10	8/10	4/10	2/10	4/10	0/10	0/10	0/10
Thickened	0/10	0/10	0/10	7/10	9/10	10/10	9/10	10/10	10/10

TABLE II

OCCURENCE OF MAJOR HISTOPATHOLOGICAL RENAL LESIONS IN MALE RATS RECEIVING CHLOROTHALONIL IN THE DIET FOR UP TO 90 DAYS (H & E STAIN)

ight Kidney)	4 Days		7 Days		2 Weeks		4 Weeks		6 Weeks		8 Weeks		10 weeks		12 Weeks		90 Days	
roup*	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
ats Used**	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
egen. Epith			1	10	1	5	2	3	5	10	3	10	3	10	9	10	7	10
pithelial yperplasia		2						5		10		10		10	1	9		10
ub. Epithel Vac.		10		10	2													
uclear Pyk.		10		10														
oss of rush Border		9		10														
ryomegaly		10		10										3	1	2		
ub. Epith. egeneration		10		10														
ub. Hyper-rophy						7		8		10				10	1	9		10
yaline Drps							10	10		10	10	9	10	10	10	10	8	10
lear Cell yperplasia																3		10

Group I = Controls
Group II = 175 mg/kg/day

* Number of rats examined histologically

TABLE III

OCCURENCE OF MAJOR HISTOPATHOLOGICAL RENAL LESIONS IN MALE RATS RECEIVING CHLOROTHALONIL IN THE DIET FOR UP TO 90 DAYS (MASON'S STAIN).

eft Kidney)	4 Days		7 Days		2 Weeks		4 Weeks		6 Weeks		8 Weeks		10 weeks		12 Weeks		90 Days	
roup *	I	II	I	II	I	II	I	II	I	II	I	II	1	II	I	II	I	II
ats Used **	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
aenol Degen		10		10		2				2		9		8		6		2
ubular yst				1		1				1			1			1		1
ubular ecrosis					10	2	10	10	10	10	10	10	10	6	10	10	10	9
ubular ctasis						10	1	3	2		1	8		10	2	9	2	5
ocus basoph ubules										5		7		8		10	2	9
ubular ast										4	1	6	4	5	6	7	9	7
nterstitial ibrosis														5		8		6

Group I = Controls
Group II = 175 mg/kg/day

* Number of rats examined histologically

TABLE IV

OCCURENCE OF MAJOR HISTOPATHOLOGICAL GASTRIC LESIONS IN MALE RATS RECEIVING CHLOROTHALONIL IN THE DIET FOR UP TO 90 DAYS.

	4 Days		7 Days		2 Weeks		4 Weeks		6 Weeks		8 Weeks		10 weeks		12 Weeks		90 Days	
roup *	I	II	I	II	I	II	I	II	I	II	I	II	1	II	I	II	I	II
ats Used **	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
quamous pithelial yperplasia		10		10		10		10		10		10		10		10		10
q.Ep.Hyper-eratosis		10		10		10		10		10		10		10		10		10
loer, Fore-tomach		10		1				4										
rosion, orestomach				4		8		9		6		4		3				2
rosion, landular tomach						3		1								1		
astritis						8		9		6		2		4				1

Group I = Controls
Group II = 175 mg/kg/day

* Number of rats examined histologically

Reviewer: D. Ritter, Toxicologist DR 5-6-88 Caswell #: 215B
Rev. Sec. # :I/Toxicology Branch
Secondary Reviewer: R. Bruce Jaeger, Section Head RBJ 5/12/88
Rev. Sec. # I/Toxicology Branch

DATA EVALUATION RECORD

Study: Determination of Co-Valent Binding of Radiolabel to DNA In The Kidneys of Male Rats Administered 14C-Chlorothalonil (14C-SDS-2787).

MRID: 40243703.

Performing Laboratory: Microbiological Associates, Inc., Bethesda, MD.

Author(s): M. C. Savides, et al.

Study ID Number: 1173-86-0096-AM-002.

Date of Study: 6/9/87.

Title: Same as above.

CORE Rating: Supplemental.

QA Statement: Satisfactory.

Conclusions: This study contains some evidence that radiolabeled Chlorothalonil does not bind co-valently to renal DNA in male mice.

METHODS:

Summary: (See Appendix A for a detailed description of the methodology used in this study).

2 Groups of 4 male Sprague-Dawley rats received either 14C-SDS-2787 (49 mg/kg) or 0.75% methylcellulose (MC) by gavage. 2 additional groups of 4 male rats each received either 14C-Dimethylnitrosamine (DMNA, 27 mg/kg, or physiological saline by intraperitoneal injection in a single dose and were sacrificed six hours after treatment. The kidneys were homogenized in an SDS-urea-phosphate buffer and the protein was precipitated with a mixture of chloroform-isoamyl alcohol-phenol (CIP). The DNA content of each kidney was determined as described in Appendix A, and the amount of bound 14C-SDS-2787 was determined.

RESULTS:

Radioactivity was found in the renal fractions of the Chlorothalonil and DMNA samples. The MC and saline samples contained little or no radioactivity. See Table I.

Of this activity, 70 to 96 % was found in the RNA fraction with the remainder being found in the DNA fraction (Table II).

The relative amounts of DNA per unit mass of kidney tissue varied w ely, over a ten-fold range (0.107 - 1.037 mg/kg tissue) The authors explained that this could be due losses during purification. See Table III.

As noted in Table IV, DMNA-treated rats had the only significant levels of renal radioactivity shown in the study. Rats receiving Chlorothalonil did not show such activity, indicating that Chlorothalonil does not bind to rat kidney DNA as does DMNA.

The high protein content of the various DNA fractions as measured by the BCA protein assay reaction were thought to be due to contamination; however, analysis using the Bio Rad Protein assay, thought to produce fewer interfering components, suggested that protein contamination was low.

DISCUSSION and CONCLUSIONS:

This study suffers from showing excessive variation in the within-group results, perhaps because of possible contamination of samples with extraneous proteins, etc. In addition, there are too few animals per dose, only 4; the authors should have used at least 6 or 8, in order to develop better statistics.

In addition, the DMNA used as the positive control should have been administered orally, the same as the Chlorothalonil, in order to show that it is exposed to the same in vivo conditions that Chlorothalonil is exposed to. Alternatively, an intraperitoneal dose of labeled Chlorothalonil could have been included.

Although the study suggests that Chlorothalonil does not bind co-valently to rat kidney DNA, it does not contain information as to whether unscheduled DNA synthesis (UDS) or replicative DNA synthesis (RDS) occurs in vivo (numerous genotoxicity studies on Chlorothalonil show that UDS does not occur in vitro). Such an in vivo assay has been developed specifically to detect UDS and RDS in male rat kidney cells that involves incorporation of tritiated thymidine into cells from rats exposed to renal toxicants and tumorigens and then analyzed autoradiographically.*

Nevertheless, the study contains evidence that Chlorothalonil does not bind co-valently to rat kidney DNA.

* Loury, D. J. and B. E. Butterworth. CIIT Act. Vol.6, No. 4. p.1.

Page_____ is not included in this copy.

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TABLE I

Specific Activity of Recovered Protein

Treatment	Animal Number	Protein mg/ml	Radioactivity DPM/ml	Specific Activity DPM/mg
MC	7851	6.52	53.00	8.13
	7852	4.17	15.00	3.60
	7853	0.85	37.10	43.65
	7867	1.24	3.00	2.42
SDS-2787	7855	2.40	4360.55	1816.90
	7856	0.73	2301.20	3152.33
	7857	0.69	1542.65	2235.72
	7858	0.59	1079.15	1829.07
Saline	7859	3.67	26.85	7.32
	7860	0.68	0.00	0.00
	7861	2.57	46.35	18.04
	7862	1.31	43.80	33.44
DMNA	7944	5.39	12206.95	2264.74
	7945	1.21	2852.35	2357.31
	7946	1.20	2033.45	1694.54
	7947	0.73	1557.10	2133.01

TABLE II

Distribution of Recovered Radioactivity

Treatment	Animal Number	Fraction	Volume	DPM per Aliquot	Total DPM per fraction	% of Total Collected
DMNA	7944	RNA	42	194.12	81530.40	68.24
			45	64.52	29034.00	24.30
			10	12.89	1289.00	1.08
		DNA	20	35.07	7014.00	5.87
			10	6.03	603.00	0.50
	7945	RNA	35	591.78	103561.50	37.16
			55	415.39	114232.25	40.98
			40	255.64	51128.00	18.34
		DNA	14	122.62	8583.40	3.08
			14	17.41	1218.70	0.44
	7946	RNA	35	703.39	123093.25	41.41
			37	551.77	102077.45	34.34
			50	247.72	61930.00	20.83
		DNA	9	36.56	1645.20	0.55
			13	100.57	6537.05	2.20
			10	39.45	1972.50	0.66
	7947	RNA	50	335.00	83750.00	44.58
			50	250.33	62582.50	33.31
			11	225.13	12382.15	6.59
		DNA	20	261.21	26121.00	13.90
			11	54.89	3018.95	1.61
SDS-2787	7855	RNA	50	386.08	193040.00	93.62
			40	30.99	12396.00	6.01
			12	1.66	199.20	0.10
		DNA	16	0.03	4.80	0.00
			16	3.43	548.80	0.27
	7856	RNA	35	870.00	152250.00	80.78
			22	235.26	25878.60	13.73
			10	154.40	7720.00	4.10
		DNA	30	14.91	2236.50	1.19
			17	4.65	395.25	0.21
	7857	RNA	39	589.70	114991.50	85.29
			15	242.18	18163.50	13.47
			13	22.37	1454.05	1.08
		DNA	15	2.27	170.25	0.13
			10	0.94	47.00	0.03
			12	0.00	0.00	0.00
	7858	RNA	40	649.81	129962.00	82.12
			39	141.33	27559.35	17.41
			7	20.24	708.40	0.45
		DNA	16	0.47	37.60	0.02
			4	0.00	0.00	0.00

TABLE III

Amounts of Recovered DNA

Treatment	Animal Number	Total DNA ug	Kidney wt g	Relative DNA mg/g
MC	7851	1411.5	2.8252	0.500
	7852	1348.4	2.6954	0.500
	7853	870.1	2.4830	0.350
	7867	1402.3	2.4529	0.572
SDS-2787	7855	3153.2	3.0402	1.037
	7856	1646.4	2.3954	0.687
	7857	391.2	2.7418	0.143
	7858	1285.7	2.7774	0.463
Saline I P	7859	1373.6	2.7846	0.493
	7860	2083.7	3.1382	0.664
	7861	2885.1	2.9703	0.971
	7862	1515.4	2.7825	0.545
DMNA I P	7944	289.2	2.6963	0.107
	7945	759.4	2.2679	0.335
	7946	600.0	2.8461	0.211
	7947	956.6	2.5539	0.375

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	7861	2885.1	2.9703	0.971
	7862	1515.4	2.7825	0.545
DMNA 1 p	7944	289.2	2.6963	0.107
	7945	759.4	2.2679	0.335
	7946	600.0	2.8461	0.211
	7947	956.6	2.5539	0.375

TABLE IV

Specific Activity of Recovered DNA

Treatment	Animal Number	DNA ug/ml	Radioactivity DPM/ml	Specific Activity DPM/mg	Protein Content ug/ml
MC	7851	346.29	0.50	1.44	51.2
	7852	391.49	1.12	2.86	131.1
	7853	262.77	0.00	0.00	558.6
	7867	467.43	16.10	34.44	216.8
SDS-2787	7855	438.30	0.00	0.00	25.3
	7856	536.17	0.00	0.00	31.1
	7857	107.45	0.00	0.00	1582.0
	7858	428.57	1.60	3.73	196.1
		377.62			
Saline	7859	420.85	0.50	1.16	260.9
	7860	336.17	3.04	9.04	231.1
	7861	961.70	8.98	9.34	40.4
	7862	504.14	5.78	11.47	148.8
DMNA	7944	71.43	39.08	547.11	44.0
	7945	230.85	257.70	1116.31	267.2
	7946	200.00	143.56	717.80	41.8
	7947	318.86	417.04	1307.91	110.9
		205.29			

CIIT ACTIVITIES
Chemical Industry Institute of Toxicology

Vol. 6, No. 4 April, 1986

Kidney-Specific DNA Repair Assay: An Evaluation of Unleaded Gasoline

David J. Loury and Byron E. Butterworth
Department of Genetic Toxicology

Unleaded gasoline has been found to cause kidney tumors in male rats. Kidney-specific short-term tests developed by CIIT's genetic toxicologists suggest that this agent — a mixture of hundreds of hydrocarbons— is not directly genotoxic in the kidney, but rather forces cell division by a toxic mechanism unique to the male rat. Intensive interdepartmental research at CIIT during the coming year will approach the mechanism of gasoline-induced kidney damage from several angles.

Each year more than 100 billion gallons of motor fuel are used by the American driving public (U.S. Bureau of Census, 1984). Unleaded gasoline, formulated for today's low-emission automobiles, represents a major proportion of this total. Because of the large number of people who regularly come into contact with unleaded gasoline or its vapors, there is considerable interest in its potential for causing adverse health effects in humans.

In the late 1970's the American Petroleum Institute initiated investigations into the subchronic and chronic toxicity of motor fuels. The long-term exposure studies, which began late in 1978 and ended in 1980, were performed on an EPA reference blend (PS-6) of unleaded gasoline with the benzene content adjusted to 2 percent. Rats and mice of both sexes were exposed by inhalation to unleaded gasoline at concentrations up to 2056 ppm for 6 hours per day, 5 days a week. At the conclusion of the study a significant, dose-dependent increase in the number of tumors in the kidneys of male rats was observed (MacFarland, 1982; Kitchen, 1984). No exposure-related increase in kidney tumors was noted in female rats or mice of either sex.

Manifestations of nephrotoxicity, observed in male rats after 3 to 12 months of exposure, included an increase of regenerative epithelium in the renal cortex and the presence of tubular dilation associated with an accumulation of intracellular protein (hyaline) droplets (Busey and Cockrell, 1984). No indication of nephrotoxicity was evident in female rats or in either sex of mice. The obvious parallels between the sex- and species-dependent patterns of renal neoplasms and renal toxicity has led to the suggestion that these two phenomena are linked mechanistically. Recent studies have suggested, however, that the male rat is uniquely susceptible to hydrocarbon-induced nephrotoxicity (Alden *et al.*, 1984), and thus may represent an inappropriate animal model for assessing human cancer risk.

Mechanisms of Action

The mechanisms involved in gasoline-induced renal neoplasia are presently the focus of an interdepartmental research project at CIIT. This study, conducted in the laboratories of Drs. James Bus, James Swenberg, and Byron Butterworth, has been divided into three major areas of emphasis: biochemistry, pathology, and genotoxicity. This report addresses pertinent findings related to the genotoxic and cytotoxic potential of unleaded gasoline in the kidney.

Carcinogens are often divided into two functional categories, genotoxic and non-genotoxic (Weisburger and Williams, 1980). Compounds with direct genotoxic activity interact with and alter DNA, whereas non-genotoxic carcinogens exert their activity by mechanisms that have not yet been clearly defined. One activity that is often seen with non-genotoxic carcinogens is forced cell proliferation. This can occur as regenerative growth following a toxic insult or can result from a direct mitogenic stimulation.

Gasoline is estimated to contain as many as 1200 to 1500 different types of hydrocarbons (Domask, 1984). One possible explanation for its carcinogenic activity may be that one or more components or component metabolites induce some form of genetic damage in the kidney. Efforts at CIIT to determine the genotoxic potential of unleaded gasoline (PS-6) using *in vitro* systems have yielded equivocal results. In tests using a human lymphoblast cell line, unleaded gasoline failed to elicit an

(Continued on page 3)

Unleaded Gasoline (from page 1)

increase in mutation frequency or to induce sister chromatid exchanges (Richardson *et al.*, 1986). However, when the same test blend was added to primary cultures of rat hepatocytes, a dose-dependent increase in DNA repair was observed (Loury *et al.*, 1986). When the same experiments were performed with mouse or human hepatocytes in culture, the cells were killed before a concentration was achieved that had produced DNA repair in the rat cells. As is so often the case, it was clear that in order to properly evaluate the genotoxic potential of this agent, activity would have to be measured in the target organ of the intact animal.

Several compounds capable of inducing renal neoplasia in laboratory animals appear to have little or no mutagenic activity *in vitro* (Kluwe *et al.*, 1984). In many instances the carcinogenic activity of these compounds is preceded by or parallels manifestation of nephropathy within the renal tubule epithelium (Goyer *et al.*, 1981; Kluwe *et al.*, 1984; Alden and Kanerva, 1982; Fowler *et al.*, 1980; National Toxicology Program, 1983). Recurrent cytotoxicity, leading to an increase in cell replication activity, has been proposed by Stott *et al.* (1981) as a possible mechanism of action for some non-genotoxic compounds. An increase in replicative DNA synthesis resulting from chemically induced nephropathy may play a role in renal carcinogenesis by converting pre-mutagenic chemical-DNA adducts to heritable mutations or by increasing the generation of spontaneous genetic errors. Recent studies have indicated that chemically induced nephrotoxicity may also promote the development of previously "initiated" kidney tumors (Shirai *et al.*, 1984).

Rationale for Developing a Kidney-Specific Assay

Aside from the issue of gasoline-induced renal carcinogenesis, there are questions concerning other environmental factors that may contribute to the development of kidney neoplasms in humans. Deaths in the United States attributable to cancer of the kidney occur at a rate of over 7,000 per year (National Cancer Institute, 1982). It is possible that some of these cases result from exposure to chemicals that are either genotoxic or cytotoxic to the renal epithelium. Experiments with laboratory animals have identified a growing number of structurally diverse renal carcinogens, including various nitrosamines, nitrilotriacetic acid, analgesics, aflatoxin B_1 (AFB_1), basic lead acetate, and N-(4'-fluoro-4-biphenylyl) acetamide. Therefore, a need exists for short-term tests capable of detecting these potential human renal carcinogens.

Since the genotoxic and cytotoxic activities of carcinogens in *in vitro* test systems may not always reflect the *in vivo* situation, it is important to use, whenever possible, whole-animal test systems for evaluating the genotoxicity and cytotoxicity of a test agent. Studies at CIIT have demonstrated that DNA repair and DNA replication induced *in vivo* by hepatocarcinogens can be detected in primary hepatocyte cultures as unscheduled DNA synthesis (UDS) and replicative DNA synthesis (RDS), respectively (Mirsalis *et al.*, 1982). In these kinds of studies the chemical is administered to the animal and then primary cultures of cells from the key target organs are prepared. These cultures are incubated with radioactive thymidine (a building block of DNA). If the DNA had been damaged by the chemical in the whole animal, it will be repaired in culture, resulting in the incorporation of the thymidine. The radioactivity is detected in the cell nucleus by autoradiography. In this process, silver grains in a photographic emulsion are produced over radioactive areas in the cell. DNA repair is thus quantitated by using a microscope to actually count the exposed silver grains over the nucleus of each cell. One advantage of the assay is that cells replicating their DNA in preparation for cell division are easly distinguished by the dense labeling pattern over the nucleus. Thus, the assay provides information on both genotoxic activity as well as forced cell proliferation. The application of this *in vivo/in vitro* method for assessing chemically induced genotoxicity and/or cell replication has been extended by CIIT and other laboratories to various tissues, including the pancreas, the nasal and tracheal epithelium, and male germ cells. In this article we describe the development, validation, and implementation of an *in vivo/in vitro* kidney cell UDS assay similar to the one reported recently by Tyson and Mirsalis (1985).

Assay Development

In assays designed to detect UDS by the autoradiographic quantitation of [^{3}H]thymidine incorporation into nuclear DNA, it is essential to obtain preparations of isolated single cells in order to assure adequate penetration of the radiolabeled material. To this end, kidney cells were isolated from treated and untreated rats by a modification of a method reported by Jones and associates (1979). Histochemical staining methods identified 40 to 55 percent of the isolated cells as originating from the proximal tubule epithelium.

To examine the metabolic activation and excision repair capabilities of isolated kidney cells, a variety of mutagenic and pro-mutagenic (requiring activation) compounds were added directly to suspensions and cultures prepared from untreated rats. Dose-related increases in UDS activity were observed with methylmethane sulfonate (MMS), ethylmethyl sulfonate (EMS), aflatoxin B_1 and 1,6-dinitropyrene in both suspensions and cultures. However, no UDS was observed with the potent kidney carcinogen dimethylnitrosamine (DMN), suggesting that once in culture kidney cells lose much of their ability to bioactivate this compound. Benzo(a)-pyrene and 2- acetylaminofluorene also produced no detectable UDS following *in vitro* administration.

To evaluate the *in vivo/in vitro* kidney cell assay for its ability to detect kidney carcinogens, the repair response to seven compounds with genotoxic activity and a proven ability to induce carcinogenesis in the kidney were examined (Table 1). In addition, the non-carcinogen diethylamine was tested. Of the kidney carcinogens tested in the *in vivo/in vitro* assay, all but AFB_1 produced statistically significant ($p \leq 0.01$) increases in repair grain counts (Table 1). The negative control compound DEA failed to elicit an increase in either total repair or net nuclear grain counts. Repair responses resulting from multi- and single-dose exposures to ethylhydroxyethylnitrosamine were compared. When given as 5 separate treatments of 500 mg/kg during a period beginning 72 hours prior to sacrifice, repair activity more than doubled that produced by a single 1000 mg/kg exposure.

In the kidney, increased RDS represents a sensitive and readily quantified indicator of focal cellular necrosis (Laurent *et al.*, 1983). Increases in RDS can be measured with the *in vivo/in vitro* kidney cells assay by determining the percentage of S-phase cells present. Cells in S-phase are readily distinguished in the assay by a high density of silver grains over the nucleus.

(Continued on page 4)

Unleaded Gasoline (from page 4)

Significance

At the present limits of detection, no DNA repair activity was observed in the kidney cell UDS assay following exposure to unleaded gasoline. These results indicate that unleaded gasoline is not directly genotoxic to the kidney. An increase in RDS activity following exposure to unleaded gasoline was seen exclusively in the kidney of the male rat. Taken together, these data suggest that unleaded gasoline has little, if any, ability to initiate tumorigenesis in the kidney, but rather, may promote the development of spontaneously initiated tumors by mechanisms related to cell turnover. Furthermore, our findings indicate that cell proliferation can be stimulated by unleaded gasoline administered by gavage as well as by inhalation, suggesting that long-term oral exposure to unleaded gasoline would probably induce kidney tumors in the male rat.

Research at CIIT is focusing on the reasons why the male rat may be uniquely susceptible to the kidney toxicity of agents such as unleaded gasoline. Studies are also currently underway at CIIT to determine the tumor promoting potential of unleaded gasoline in the kidney of male and female rats. These experiments will take advantage of kidney tumor initiation/promotion protocols recently developed by American and Japanese investigators. During the course of this year-long investigation, efforts will be made to correlate cell replication activity of unleaded gasoline with tumor promoting potential.

The Authors

Dr. Loury (above) received his Ph.D. in pharmacology and toxicology from the University of California—Davis in 1984, and began a CIIT postdoctoral fellowship in the Department of Genetic Toxicology the same year. He is an associate member of the Society of Toxicology and the Environmental Mutagen Society.

Dr. Butterworth has been chief of genetic toxicology at CIIT since 1977.

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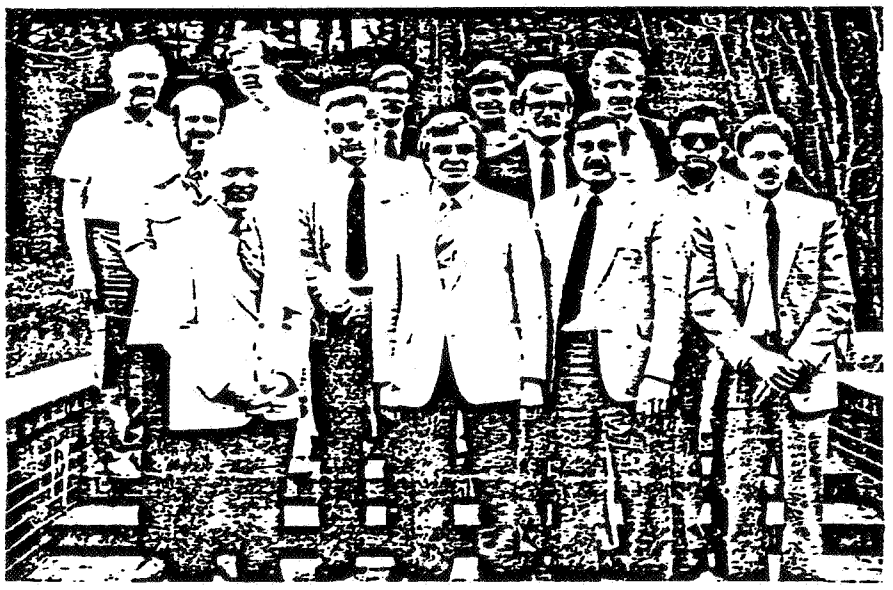
Continued on page 7)

Dr. Mirkes (above, left) of the Central Laboratory for Human Embryology at the University of Washington, discusses molecular aspects of cyclophosphamide teratogenesis with Nigel Brown, Medical Research Council, United Kingdom. Dr. Brown spoke on the teratogenic mechanisms of valproate, 2-methoxyacetate, and other carboxy... acids.



The speakers, left to right:

Bottom row: Drs. William J. Scott, Jr., University of Cincinnati; Frank Welsch, CIIT (conference organizer); Philip E. Mirkes, University of Washington; and Nigel Brown, Experimental Embryology and Teratology Unit, Medical Research Council, United Kingdom.

Middle row: Drs. Robert M. Pratt, National Institute of Environmental Health Sciences; Mont Juchau, University of Washington; Randolph B. Sleet, CIIT; and Devendra M. Kochhar, Jefferson Medical College.

Top row: Drs. Diether Neubert, Free University of Berlin; Oliver P. Flint, Imperial Chemical Industries, United Kingdom; Walter E. Horton, National Institute of Dental Research; Michael H. L. Snow, Mammalian Development Unit, Medical Research Council, United Kingdom; and Heinz Nau, Free University of Berlin.

Speakers not pictured: Dr. Thomas E. Sadler, University of North Carolina at Chapel Hill; Dr. Jeanne Manson, Smith, Kline & French Laboratories; and Dr. Robert A. Brent, Jefferson Medical College.

Unleaded Gasoline (from page 5)

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